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14. ABSTRACT

During the performance period, we successfully constructed an N-terminal serine to cysteine mutant of PlyCB. This mutant was expressed, purified, reacted with gold nanoparticles and the resulting PlyCB/gold nanoparticle complex was then purified. A fraction of the complex was further conjugated to a fluorescent dye and specific binding of the complex to streptococcal cells, but not non-target cells, was observed by both bright field and fluorescent microscopy. Next, the nanoparticles were exposed to various power settings on an infrared spectrophotometer and showed a measured response to input power. In parallel we tested the viability of streptococcal cells at different temperatures and at different exposure times. Finally, we have cloned a K. pneumococcus binding domain and are making fusions to the C-terminus of PlyCB in order to determine if we can switch specificity of the PlyCB/gold nanoparticle complex toward other pathogens.

15. SUBJECT TERMS
Gold, Nanoparticle, Infrared Radiation, Infrared Spectroscopy, Thermal Ablation, Bacteriophage

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INTRODUCTION:

Due to the changing bacteriology and multi-drug resistant nature of osteomyelitis (bone infections) associated with war wounds, choosing appropriate antimicrobial therapy is a challenging task (Calhoun, 2008; Johnaon, 2007; Murray, 2006; Murray, 2008; Yun, 2008). One alternative approach to antibiotics includes the use of near infrared (IR) radiation to thermally kill pathogenic organisms (Kam, 2005; Zharov, 2006). Whereas near IR wavelengths pass harmlessly through the human body, they are known to heat gold nanoshells to high temperatures (>70°C), which in principle will thermally ablate any cell (bacterial or eukaryotic) in close proximity to these particles. Due to the rapid dissipation of thermal energy over very short distances, it is speculated that only cells in direct contact with gold particles will be killed and surrounding tissues will be unaffected (Hu, 2006). The challenge to this approach is to develop a system to bind the gold nanoparticles to the surface of targeted cells. Our aim is to use a scaffold protein, PlyCB, which can be functionalized to bind gold nanoparticles as well as bacterial-specific targeting domains. PlyCB is stable up to 100°C, resistant to proteolysis, high ionic strength, extreme pH conditions, and most detergents, making it a perfect platform for engineering. The "top" surface of PlyCB will be modified to contain cysteine residues to covalently bind gold nanoparticles (Fig 1). The "bottom" surface of PlyCB inherently binds streptococcal cells with nanomolar affinity, which will be used for proof-of-principle thermal ablation studies. We further propose to engineer the "bottom" surface to contain an array of binding domains isolated from tail fibers of bacteriophage that are specific for osteomyelitis causing pathogens.

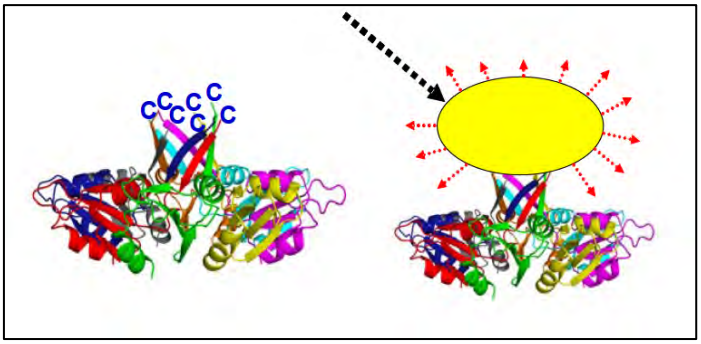


Fig. 1. Left, PlyCB with a Ser to Cys mutation at the N-terminal amino acid. Right, gold particles will bind PlyCB via the cysteine residues, making the PlyCB/gold nanoparticle complex that can be used for thermal ablation studies.

BODY:

Aim 1. Make PlyCB/Gold nanocomplexes.

Task 1. Obtain reagents. (month 1)

All reagents have been obtained. The gold nanoparticles causes us the most trouble. Initially, we were using 100 nm gold nanoparticles (round balls). However, after fine tuning our infrared spectrophotometer and receiving more technical advice from the vendor, we found that a nanorod, not a sphere, produced better surface plasmon resonance for our applications. We now have normal nanorods as well as neutravidin-coated rods.

Task 2. Make PlyCB mutations (change serine at position 1 to cysteine (S1C mutant). (month 1-3)

The single point mutant (Ser at position 1 to cystein e) was easily accomplished by a QuickChange Mutagenesis kit. The S1C mutant was expressed, purified, and confirmed to contain the mutation by both mass spectral analysis and N-terminal sequence analysis (data not shown). Additionally, we confirmed by analytical gel filtration that the mutant had no adverse affect on formation of the PlyCB octamer. We were able to generate ~50 mg of pure S1C mutant from a 6L fermentation.

Task 3. Make PlyCB/gold nanoparticle complex. (month 4-9)

Initially, 1 mg of the S1C mutant of PlyC was reacted with ~1x10 ^{10} gold nanoparticles (nps)/ml and purified by size exclusion chromatography (Fig. 2). The chemistry involved relied on free, reduced sulfhydral moieties of the cysteine residues to interact directly with the gold. This approach was very successful at forming the complex. However, the complexes were "sticky" and would often bind to the container they were stored in or they would aggregate and fall out of solution. To solve this problem, we switched to using gold nanoparticles that were coated with a neutravidin shell. In order to bind to the neutravidin, we biotinylated the cysteine residues of the S1C mutant. Although this was a setback, we did achieve formation of the complex, were able to purify it, and successfully store it without aggregation issues (data not shown).

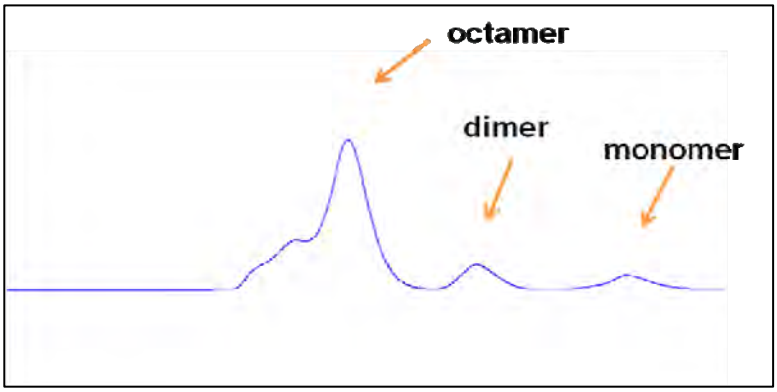


Fig. 2. Analytical gel filtration showing formation of the PlyCB octamer complexed to the gold nanoparticles.

Another issue we encountered (later in Aim 2), caused us to return to this task. Namely, the 100 nm spherical gold nanoparticles we were using were not optimized for thermal ablation by near infrared spectroscopy. Through trial and error, we determined a gold nanorod, roughly 10 nm x 30 nm, gave the best surface plasmon resonance for thermal ablation studies by infrared radiation. Thankfully, the vendor made neutravidin rods in the size/length we required. We now have a stock of PlyCB/gold nanorod complexes for future studies.

Next, we chemically crosslinked the complex with a fluorophore (AlexaFluor 488), mixed it with streptococcal cells, washed, and viewed under bright field and fluorescent microscopy. As can be seen in Fig. 3, gold nanoparticles adhered to the surface of the streptococci. We wanted to determine if this binding event was specific for the streptococcal surface or simply an artifact of a "sticky" complex. Therefore, we repeated the experiment in the presence of both streptococci and *E. coli*, a non-target host for PlyCB. As can be seen in Fig. 4, the PlyCB/gold nanoparticle complex specifically binds streptococci.

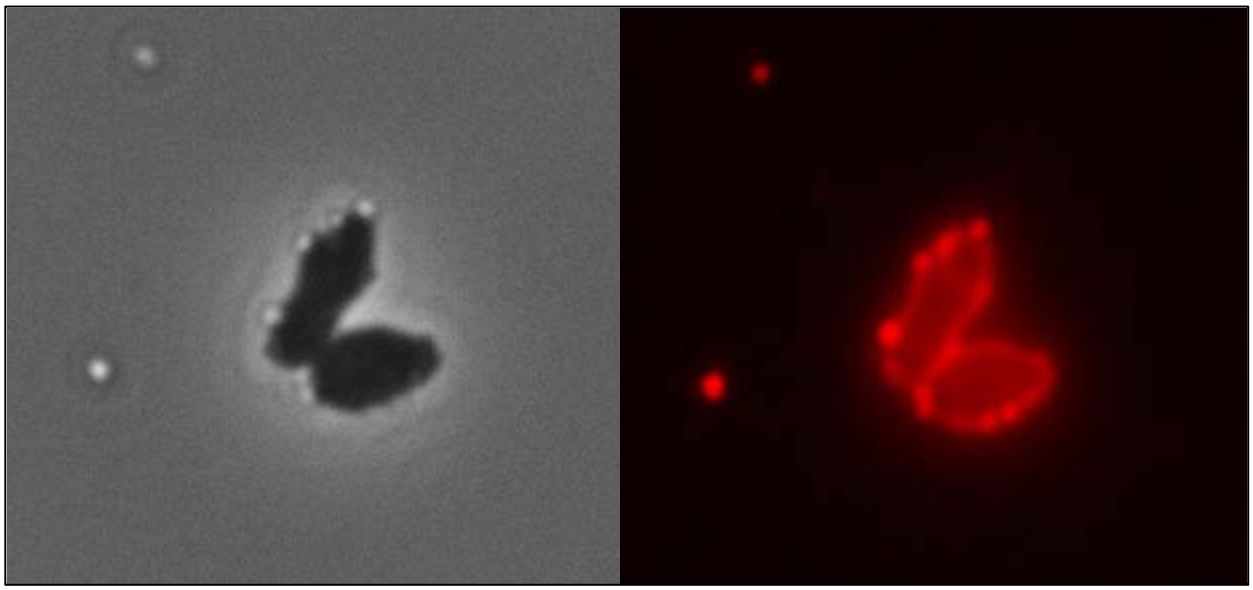
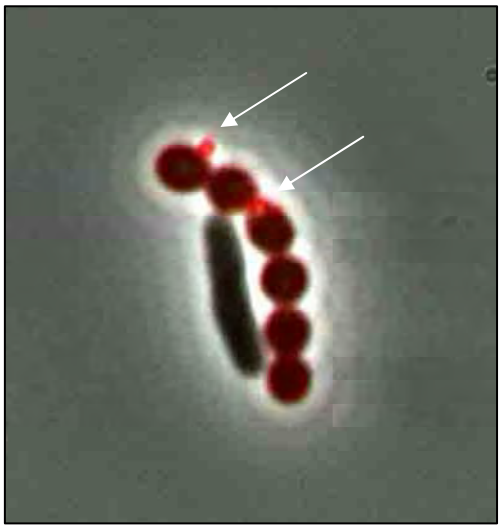


Fig. 3. Left, bright field image of PlyCB/gold nanoparticle complexes binding streptococcal cells. The 100 nm gold spheres coated with PlyCB can be seen as white dots on the streptococcal cell. Right, fluorescent image. PlyCB was labeled with a fluorophore before being complexed to the gold nanoparticles. As such, the particles can be seen as bright red spheres.

Fig. 4. The same experiment as in Figure 3, but this time, E. colie was mixed with streptococci. The PlyCB/gold complexes specifically bind streptococci (white arrows) but not the E. coli.



Aim 2. Proof-of-principle thermal ablation experiments.

Task 1. Irradiation and power tests. (month 9-10)

Before we can perform irradiation and power test on our nanocomplexes, we needed to do some basic studies on how much heat is needed to kill streptococci and at what duration. Therefore, we heated streptococci to various temperatures for different amounts of time. As shown in Fig. 5, all streptococci (i.e. 10^7) were killed when exposed to 50°C for 10 minutes. However, when

the temperature was raised to 60°C, only 10 seconds was needed to produce a 4 log drop in viability and 65°C sterilized the culture. Given that thermal ablation can produce very high temperatures, we may only need a fraction of a second of contact time.

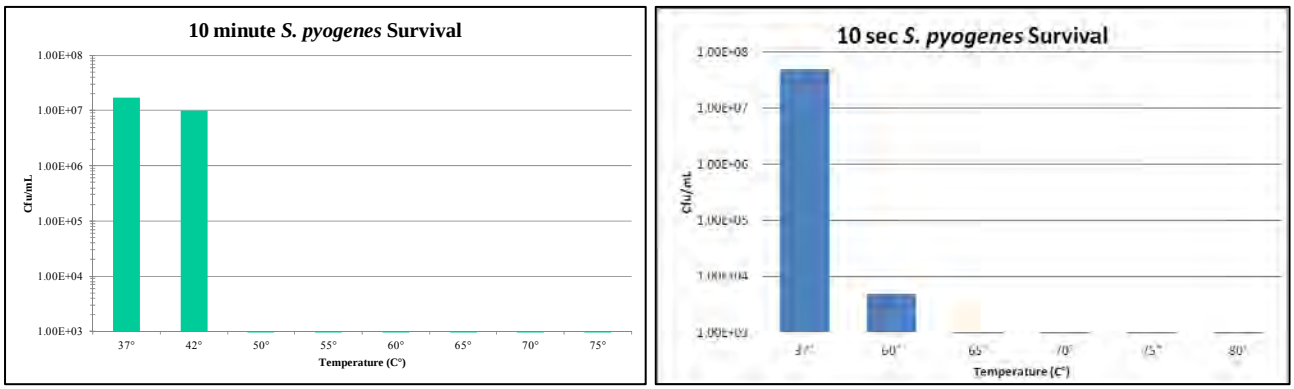


Fig. 5. Left, thermal killing after 10 min exposure. Right, thermal killing after 10 second exposure.

Finally, we were ready for power testing. As can be seen in Fig. 6, just 5 or 10 milijules of laser power was sufficient to produce a strong resonant energy. We are may be sufficient for thermal ablation studies.

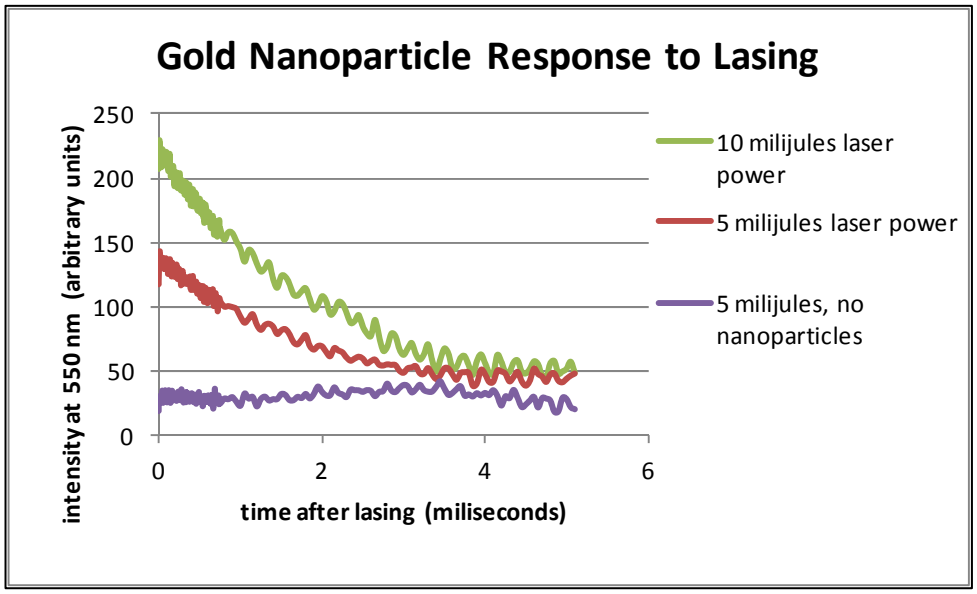


Fig. 6. Resonance energy response of PlyCB/gold nanoparticle complex to near infrared lasing at 550 nm.

Task 2. Bacterial ablation testing. (month 11-13)

In our initial bacterial ablation experiments, it appeared as if thermal ablation of streptococci worked at all power settings (data not shown). However, we noticed that controls where infrared lasing did not occur also produced a significant drop in viability. Therefore, we had immediate

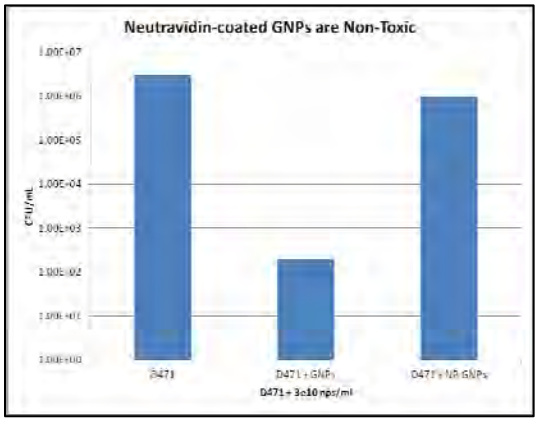


Fig. 7. "Bare" gold nanoparticles was toxic to streptococcal strain D471, but when the same particles were coated with neutravidin, toxicity was avoided.

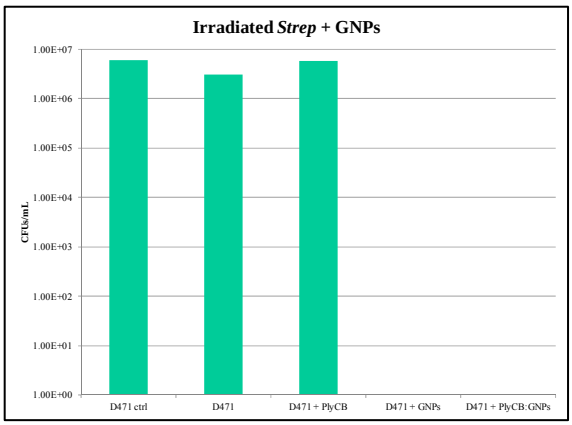


Fig. 8. $6x10^6$ colony forming units/ml of streptococcal strain D471 was mixed with $6x10^{10}$ nanoparticles/ml and irradiated with 50 pulses of a 5 mJ laser at 569 nm. Only wells containing gold nanoparticles were sterilized.

concerns that the gold nanoparticles themselves were toxic to streptococci. Indeed, as seen in Fig. 7, bare gold nanoparticles were somewhat toxic to the bacteria in the absence of near infrared lasing. However, the neutravidin-coated particles were found to be non-toxic. Current/future experiments will only use the neutravidin-coated particles bound to the biotinylated S1C mutant of PlyCB. Significantly, our most recent data does show thermal ablation of streptococcal strain D471 by neutravidin gold nanoparticles both alone and complexted with PlyCB (Fig. 8).

Aim 3. Engineer bacteriophage tail fiber domains.

Task 1. Obtain and characterized bacteriophage. (month 1-6)

We have obtained several phage through depositories or international collaborators. Sequencing has been an issue and even when sequencing is available, there is a bit of a bio informatic bottleneck trying to elucidate which domains on a tail fiber are responsible for binding to the bacterial surface and which domains are structural. Nonetheless, we have identified a binding domain on a pneumococcal phage that we believe is responsible for binding to the surface. This will serve us for our proof of concept studies.

Task 2. Make PlyCB/gold nanoparticle constructs with selected phage tail fiber or lysin binding domains. (month 7-12)

As stated above, we have identified a pneumoccal binding domain for proof of principle experiments, synthesized this gene, and made a fusion of this gene to the C-terminal domain of PlyCB (data not shown). We are currently in the process of expressing this chimera and evaluating its solubility.

KEY RESEARCH ACCOMPLISHMENTS:

- Successfully created S1C mutation of PlyCB
- Made PlyCB/gold nanoparticle complexes
- Demonstrated that PlyCB/gold nanoparticle complexes retain specific binding to streptococcal cells
- Overcame hurdles with respect to size/shape of gold nanoparticle and toxicity of uncoated gold nanoparticles
- Demonstrated resonance energy output from nanoparticles lased with near infrared radiation
- Demonstrated thermal ablation of streptococci with PlyCB/gold nanoparticle complexes
- Created a chimeric fusion of PlyCB with a pneumococcal binding domain

REPORTABLE OUTCOMES:

Some aspects of our prelim inary data and/or overall strategy have been presented at the following meetings/symposia during the past year:

- Nineteenth Evergreen International Phage Biology Meeting. Olympia, WA. Poster presentation titled "Thermal Ablation of Streptococci using an Endolysin Binding Domain as a Functionalized Nanoparticle"
- Georgia Institute of Technology, Atlanta, GA. Seminar speaker.
- Catholic University, Washington, D.C. Seminar speaker.
- Rockefeller University, New York, NY. Seminar speaker.

CONCLUSION:

Our results have been consistent with our expect ations. We had several technical hurdles with the size of the gold nanoparticle and toxicity of bare nanoparticles, but we have overcom e these issues. More im portantly, we successfully made the required S1C mutation, form ation of PlyCB/gold nanoparticle com plexes was straight -forward, and as expected, the com plexes retained the species-specific binding properties of wild-type PlyCB. The final six m onths of the project will be spent further dem onstrating the thermal ablation properties of the complexes and evaluating if we can change specificity of the complex by addition of a non-host binding dom ain to the C-terminus of PlyCB (i.e. the pneumococcal binding domain).

If successful, this generalized bioengineering scheme can be applied to a wide range of pathogens.

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APPENDICES:

The following abstract was presented as a poster at the Nineteenth Evergreen International Phage Biology Meeting in August, 2011 in Olympia, Washington.

Thermal Ablation of Streptococci using an Endolysin Binding Domain as a Functionalized Nanoparticle

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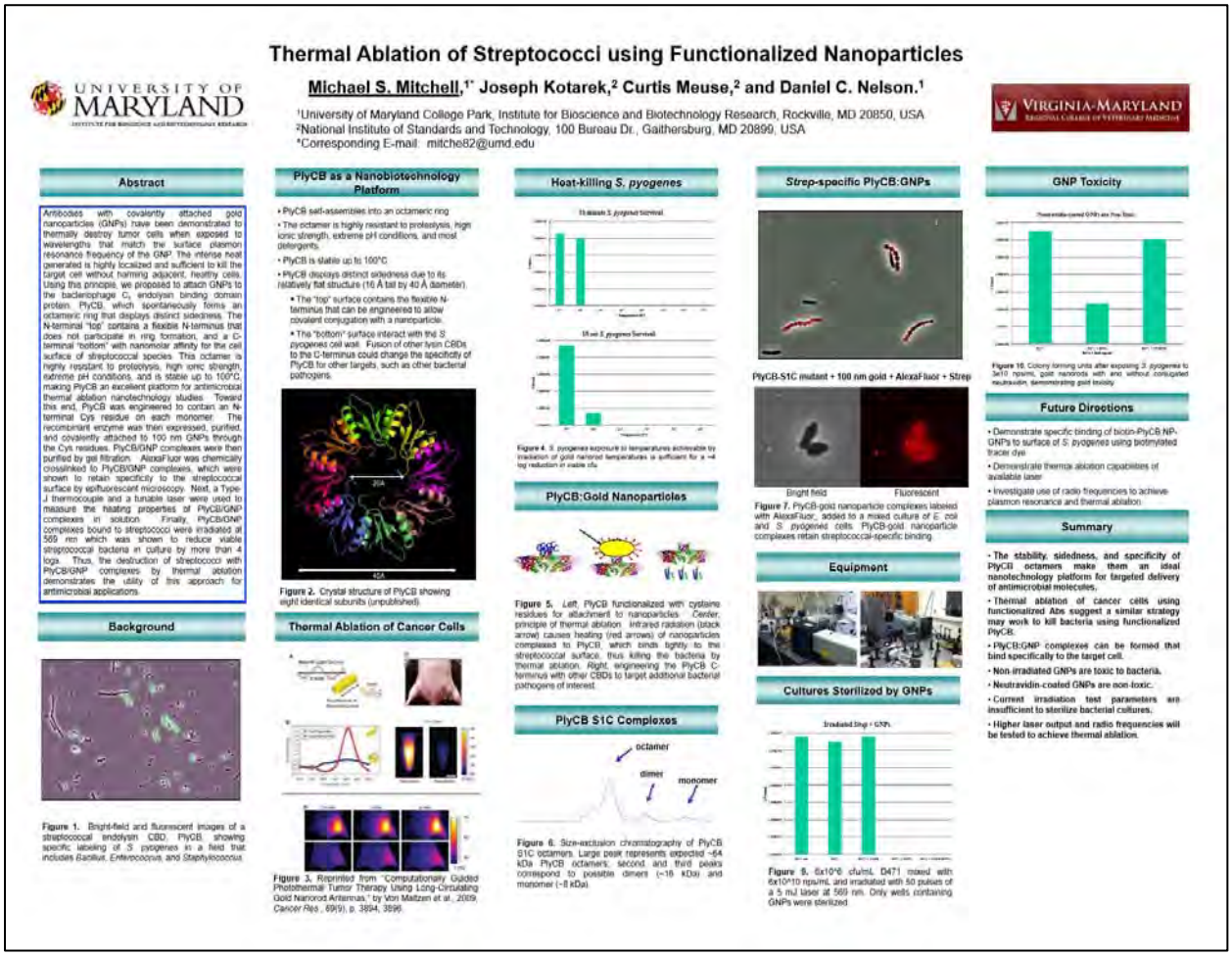
Antibodies with covalently attached gold nanoparticles (GNPs) have been demonstrated to thermally destroy tumor cells when exposed to wavelengths that match the surface plasmon resonance frequency of the GNP. The intense heat generated is highly localized and sufficient to kill the target cell without harming adjacent, healthy cells. Using this principle, we proposed to attach GNPs to the bacteriophage C1 endolysin binding domain protein, PlyCB, which spontaneously forms an octameric ring that displays distinct sidedness. The N-terminal "top" contains a flexible N-terminus that does not participate in ring formation, and a C-terminal "bottom" with nanomolar affinity for the cell surface of streptococcal species. This octamer is highly resistant to proteolysis, high ionic strength, extreme pH conditions, and is stable up to 100°C, making PlyCB an excellent platform for antimicrobial thermal ablation nanotechnology studies. Toward this end, PlyCB was engineered to contain an N-terminal Cys residue on each monomer. The recombinant enzyme was then expressed, purified, and covalently attached to 100 nm GNPs through the Cys residues. PlyCB/GNP complexes were then purified by gel filtration. AlexaFluor was chemically crosslinked to PlyCB/GNP complexes, which were shown to retain specificity to the streptococcal surface by epifluorescent microscopy. Next, a Type-J thermocouple and a tunable laser were used to measure the heating properties of PlyCB/GNP complexes in solution. Finally, PlyCB/GNP complexes bound to streptococci were irradiated at 569 nm which was shown to reduce viable streptococcal bacteria in culture by more than 4 logs. Thus, the destruction of streptococci with PlyCB/GNP complexes by thermal ablation demonstrates the utility of this approach for antimicrobial applications.

A copy of the poster is follows:



Thermal Ablation of Streptococci using Functionalized Nanoparticles

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Abstract

Antibodies with covalently attached gold nanoparticles (GNPs) have been demonstrated to thermally destroy tumor cells when exposed to wavelengths that match the surface plasmon resonance frequency of the GNP. The intense heat generated is highly localized and sufficient to kill the target cell without harming adjacent, healthy cells. Using this principle, we proposed to attach GNPs to the bacteriophage C_1 endolysin binding domain protein, PlyCB, which spontaneously forms an octameric ring that displays distinct sidedness. The N-terminal "top" contains a flexible N-terminus that does not participate in ring formation, and a C-terminal "bottom" with nanomolar affinity for the cell surface of streptococcal species. This octamer is highly resistant to proteolysis, high ionic strength, extreme pH conditions, and is stable up to 100°C, making PlyCB an excellent platform for antimicrobial thermal ablation nanotechnology studies. Toward this end, PlyCB was engineered to contain an N-terminal Cys residue on each monomer. The recombinant enzyme was then expressed, purified, and covalently attached to 100 nm GNPs through the Cys residues. PlyCB/GNP complexes were then purified by gel filtration. AlexaFluor was chemically crosslinked to PlyCB/GNP complexes, which were shown to retain specificity to the streptococcal surface by epifluorescent microscopy. Next, a Type-J thermocouple and a tunable laser were used to measure the heating properties of PlyCB/GNP complexes in solution. Finally, PlyCB/GNP complexes bound to streptococci were irradiated at 569 nm which was shown to reduce viable streptococcal bacteria in culture by more than 4 logs. Thus, the destruction of streptococci with PlyCB/GNP complexes by thermal ablation demonstrates the utility of this approach for antimicrobial applications.

Background

Figure 1. Bright-field and fluorescent images of a streptococcal endolysin CBD, PlyCB, showing specific labeling of *S. pyogenes* in a field that includes *Bacillus*, *Enterococcus*, and *Staphylococcus*.

PlyCB as a Nanobiotechnology Platform

- PlyCB self-assembles into an octameric ring.
- The octamer is highly resistant to proteolysis, high ionic strength, extreme pH conditions, and most detergents.
- PlyCB is stable up to 100°C.
- PlyCB displays distinct sidedness due to its relatively flat structure (16 Å tall by 40 Å diameter).
  - The "top" surface contains the flexible N-terminus that can be engineered to allow covalent conjugation with a nanoparticle.
  - The "bottom" surface interact with the *S. pyogenes* cell wall. Fusion of other lysin CBDs to the C-terminus could change the specificity of PlyCB for other targets, such as other bacterial pathogens.

Figure 2. Crystal structure of PlyCB showing eight identical subunits (unpublished).

Thermal Ablation of Cancer Cells

Figure 3. Reprinted from "Computationally Guided Photothermal Tumor Therapy Using Long-Circulating Gold Nanorod Antennas." by Von Maltzen et al., 2009, *Cancer Res.*, 69(9), p. 3894, 3896.

Heat-killing *S. pyogenes*

Figure 4. *S. pyogenes* exposure to temperatures achievable by irradiation of gold nanorod temperatures is sufficient for a >4 log reduction in viable cfu.

PlyCB:Gold Nanoparticles

Figure 5. *Left*, PlyCB functionalized with cysteine residues for attachment to nanoparticles. *Center*, principle of thermal ablation. Infrared radiation (black arrow) causes heating (red arrows) of nanoparticles complexed to PlyCB, which binds tightly to the streptococcal surface, thus killing the bacteria by thermal ablation. *Right*, engineering the PlyCB C-terminus with other CBDs to target additional bacterial pathogens of interest.

PlyCB S1C Complexes

Figure 6. Size-exclusion chromatography of PlyCB S1C octamers. Large peak represents expected ~64 kDa PlyCB octamers; second and third peaks correspond to possible dimers (~16 kDa) and monomer (~8 kDa).

Strep-specific PlyCB:GNPs

PlyCB-S1C mutant + 100 nm gold + AlexaFluor + Strep

Bright field Fluorescent

Figure 7. PlyCB-gold nanoparticle complexes labeled with AlexaFluor, added to a mixed culture of *E. coli* and *S. pyogenes* cells. PlyCB-gold nanoparticle complexes retain streptococcal-specific binding.

Equipment

Cultures Sterilized by GNPs

Figure 8. 6x10^6 cfu/mL D471 mixed with 6x10^10 nps/mL and irradiated with 50 pulses of a 5 mJ laser at 569 nm. Only wells containing GNPs were sterilized.

GNP Toxicity

Figure 10. Colony forming units after exposing *S. pyogenes* to 3e10 nps/mL gold nanorods with and without conjugated neutravidin, demonstrating gold toxicity.

Future Directions

- Demonstrate specific binding of biotin-PlyCB:NP-GNPs to surface of *S. pyogenes* using biotinylated fractal dye.
- Demonstrate thermal ablation capabilities of available laser.
- Investigate use of radio frequencies to achieve plasmon resonance and thermal ablation.

Summary

- **The stability, sidedness, and specificity of PlyCB octamers make them an ideal nanotechnology platform for targeted delivery of antimicrobial molecules.**
- **Thermal ablation of cancer cells using functionalized Abs suggest a similar strategy may work to kill bacteria using functionalized PlyCB.**
- **PlyCB:GNP complexes can be formed that bind specifically to the target cell.**
- **Non-irradiated GNPs are toxic to bacteria.**
- **Neutravidin-coated GNPs are non-toxic.**
- **Current irradiation test parameters are insufficient to sterilize bacterial cultures.**
- **Higher laser output and radio frequencies will be tested to achieve thermal ablation.**

SUPPORTING DATA:

Figures and figure legends are contained within the body of the text above.